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HIGH ELECTROCATALYTIC ACTIVITY OF TETHERED MULTICOPPER OXIDASE-CARBON NANOTUBE CONJUGATES
(POSTPRINT)

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Multicopper oxidases linked to multiwall carbon nanotubes via the molecular tethering reagent, 1-pyrenebutanoic acid, succinimydl ester, diplayed high bioelectrocatalytic activity for oxygen reduction.

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High electrocatalytic activity of tethered multicopper oxidase–carbon nanotube conjugates

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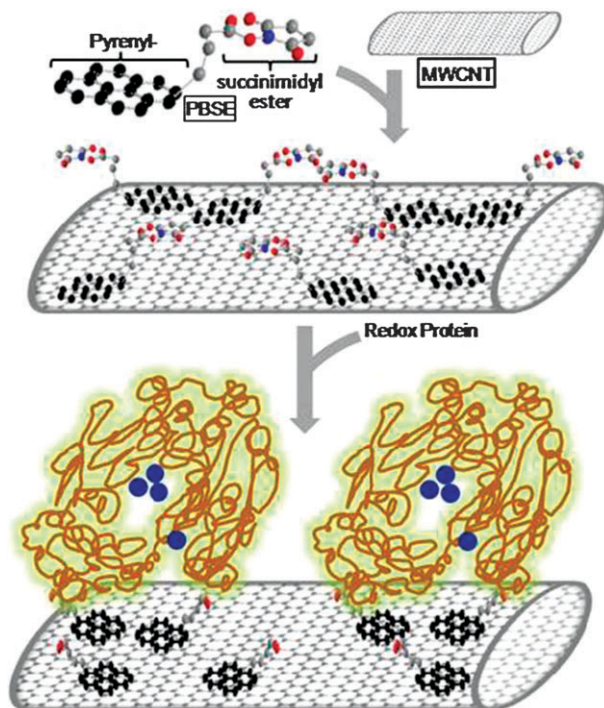Plamen B. Atanassov[c] and Glenn R. Johnson*[a]

Multicopper oxidases linked to multiwall carbon nanotubes *via* the molecular tethering reagent, 1-pyrenebutanoic acid, succinimidyl ester, displayed high bioelectrocatalytic activity for oxygen reduction.

The multicopper oxidase (MCO) enzymes catalyze oxygen reduction at high electrochemical potentials, making them attractive catalysts for biological fuel cell cathodes.[1] The redox active center of an MCO comprises four coordinated copper atoms: type 1 (T1), type 2 (T2) and type 3 (T3) copper sites.[2] The T1 site lies close to the substrate-binding pocket of the enzyme. In a catalytically active system, the T1 site is the initial electron acceptor, which then subsequently passes electrons to the tri-nuclear copper center (T2 and two T3) where oxygen is ultimately reduced to water. Various experimental strategies have been proposed for establishing direct electron transfer (DET) with enzymatic electrodes in which the electrons may 'hop' between the enzyme redox center and the electrode. The DET mode eliminates the inherent limitations of redox mediators in biological fuel cell and biosensor applications.[3] The strategies to electronically connect the electrode and biocatalysts include: simple physisorption or covalent linkage, entrapment in conductive polymeric films, association with metal colloids, and encapsulation within porous matrices that incorporate a conductive nanomaterial.[4] Immobilization methods using carbon nanotubes (CNT) are an attractive means to create a three-dimensional, porous, conductive catalytic matrix on an electrode surface.[5] Establishing a methodology that will reliably link the redox enzymes to well dispersed, conductive CNT may advance bioelectrocatalysis and materials applications.

Chen *et al.* reported that CNT can be modified using 1-pyrenebutanoic acid, succinimidyl ester (PBSE). The aromatic pyrenyl moiety interacts with the aromatic-like structure of the CNT walls through irreversible π–π stacking at the CNT and PBSE interface.[6] Subsequent incubation of the PBSE-modified CNT with protein allows the amines on the protein surface to form covalent amide bonds that link the proteins to PBSE and therefore to CNT. The PBSE-based method for sidewall functionalization and protein immobilization on CNT has been studied with various model enzymes, but little work has been related to bioelectrocatalysis and in particular, DET of redox enzymes.[7]

For an initial proof of concept, two MCO were selected as model redox enzymes, laccase from *Trametes versicolor* and bilirubin oxidase (BOx) from *Myrothecium verrucaria*. The enzymes were immobilized on multi-walled CNT (MWCNT) by PBSE-modification of MWCNT directly on Toray® carbon paper (TP) electrodes (Scheme 1).† The enzyme immobilization efficiency was examined first using standard biochemical assays.† The assay showed that ~20 U of laccase was associated with the electrode, corresponding to ~0.03 μg protein mm^{-2} electrode. BOx activity proved to be too low to measure spectrophotometrically for the corresponding BOx-modified PBSE/MWCNT electrode. Since the activity measured in the standard assay does not represent electrochemical activity or oxygen reduction, further electrochemical characterization of the materials was done to determine whether an electronic connection was established for the protein, MWCNT, and TP composite material.†

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Scheme 1 Illustration of MCO immobilization onto PBSE-modified carbon nanotubes.

Cyclic voltammetry (CV) was used to evaluate the effectiveness of the PBSE-tether for functionalizing the TP/MWCNT-electrodes with enzyme. For control experiments, enzymes were associated directly to (i) TP or (ii) TP/MWCNT through noncovalent physisorption. Laccase adsorbed directly on TP showed no evidence of electrocatalytic activity for oxygen reduction (Fig. 1a). Despite a theoretically short electron tunneling distance between the enzyme redox center and the electrode, the open circuit potential (OCP) for the oxygen reduction reaction was 0.44 ± 0.03 V; $n = 4$, significantly lower than the thermodynamic maximum (0.688 V $vs.$ Ag/AgCl) at pH 5.8. TP modified with MWCNT had higher capacitance (increased electrochemical surface area) but marginally increased OCP (0.49 ± 0.02 V; $n = 3$). Although the nanomaterial dimensions may bring about close physical binding between laccase and MWCNT that could facilitate electron tunnelling, there was no evidence for interfacial DET or electrocatalytic activity.

When laccase was tethered to MWCNT via PBSE, the CV results depicted obvious electrocatalytic activity for oxygen reduction. The cathodic sweep showed a dramatic deflection from the control electrode processes below 0.6 V. The OCP, onset and half-peak potentials were 0.60 ± 0.01 V, 0.60 ± 0.01 V,

and 0.47 ± 0.02 V; $n = 3$, respectively, and diffusion limitation conditions were reached at ~ 0.4 V during the cathodic sweep. The voltammetric response of the PBSE-tethered laccase provided a Tafel slope of 18 mV per decade in the kinetic region above 0.57 V and a slope of 24 mV per decade from 0.5 to 0.55 V. The Tafel slopes approach the theoretical limit (15 mV) for a four-electron-transfer reaction, and compare favorably with slopes for conventional oxygen reduction catalysts.[8] The CV trace for nitrogen-sparged electrolyte shows no catalytic current with the tethered laccase, confirming that the deflection seen in the cathodic sweep corresponds to oxygen reduction (Fig. 1).

The methodology was examined further using BOx as an oxygen reduction catalyst. The PBSE-tethered BOx electrode (Fig. 1b) also showed high electrocatalytic activity (OCP: 0.62 ± 0.005 V; $n = 3$), the onset and half-peak potentials for the electrodes were 0.61 ± 0.02 V and 0.45 ± 0.03 V, respectively. The OCP at neutral pH was ~ 0.56 V, about 75 mV higher than that reported in the literature for a covalently linked BOx on MWCNT.[9] The CV traces of oxygen- and nitrogen-sparged electrolyte show a clear distinction between catalytic and capacitive processes, indicating a high electrocatalytic activity of BOx. Unlike laccase, BOx exhibited apparent electrocatalytic activity after simple physisorption onto the TP or TP/MWCNT electrodes without the PBSE tether. The response may result from undefined surface characteristics of BOx that bring about preferential orientation of the enzyme T1 copper site compared to laccase, or simply a higher specific catalytic activity for the commercial BOx preparation.

Galvanostatic measurements at steady state revealed that the tethered MCO exhibit exceptionally stable performance with potential losses of less than 100 mV at 50 μA cm^{-2}, relative to the OCP. By comparison, laccase physisorbed onto TP or TP/MWCNT-modified could not sustain high faradic currents (Fig. 2). The BOx on TP and TP/MWCNT
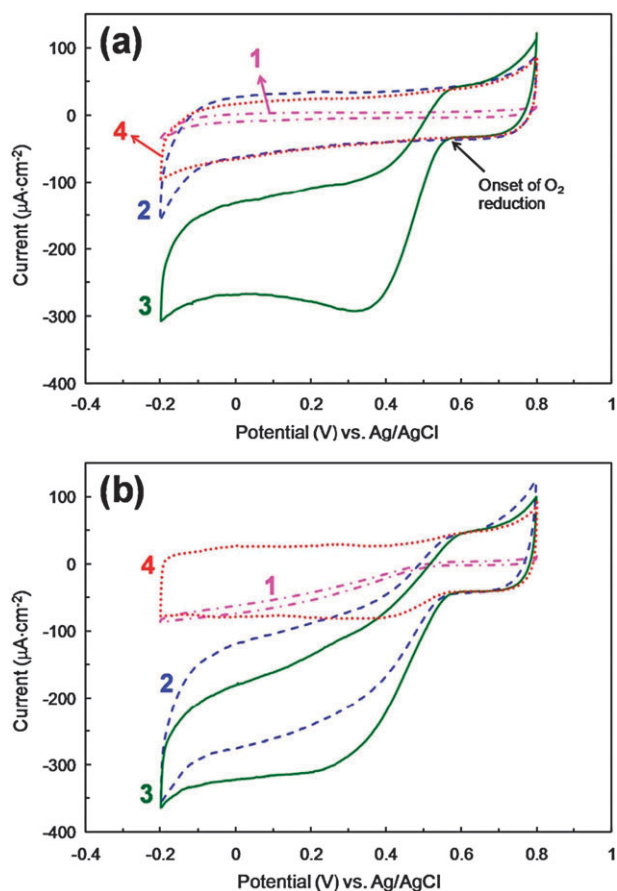
Fig. 1 Cyclic voltammograms (CV) of model MCO bioelectrodes (laccase (a), BOx (b)). Key: (1) MCO physisorbed on bare TP, (2) MCO physisorbed on TP/MWCNT electrode; (3) MCO immobilized on PBSE-modified TP/MWCNT electrode; (4) electrode 3 in nitrogen-flushed electrolyte. CV scans in phosphate buffer electrolyte (pH 5.8), scan rate 10 mV s^{-1}, oxygen saturated electrolyte except as noted.
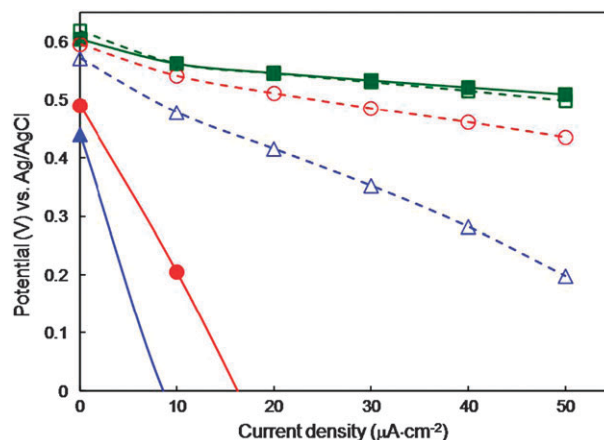
Fig. 2 Galvanostatic polarization curves for laccase (solid lines, closed symbols) and BOx (dotted lines, open symbols) electrodes. Key: (triangles) MCO physisorbed on bare TP; (circles) MCO physisorbed on TP/MWCNT electrode; (squares) MCO immobilized on PBSE-modified TP/MWCNT electrode. Note: potentials <0 are not shown; the plotted curves extrapolate beyond the x-axis and are based on all measured data.

electrodes, however, showed comparably better performance than laccase controls, in agreement with the CV results.

The PBSE-modified TP/MWCNT electrodes supported faster and more complete bioelectrochemical oxygen reduction than the physisorbed control materials and facilitated DET for immobilized MCO at current densities much greater than previous reports.[9] The galvanostatic measurements for the MCO confirm the fabrication of a stable, conductive, bio-electrocatalytic interface between the enzyme and electrode that may be attributed to a variety of factors. The PBSE-modification of MWCNT *via* π–π stacking preserves the electronic properties of the MWCNT to allow efficient electron transport through the matrix. The covalent link between a MCO and the PBSE-tether will position the enzyme close to the MWCNT and reduce the electron tunneling distance between the enzyme and the electrode. In addition, the distribution of amines on the protein surface will guide orientation with the MWCNT. The limited electrochemical activity measured for control electrodes (no PBSE) is attributed to a small fraction of MCO molecules that align favorably during physisorption on TP or MWCNT. The response is evident from the higher OCP measured with MWCNT on TP (0.49 V) when compared to that of protein-free MWCNT (0.09 V). The high OCP, however, did not translate to electrocatalytic activity, possibly due to unfavorable orientation of the majority of catalyst molecules on the MWCNT surface or poor catalyst loading. A detailed understanding of the biophysical interactions at the interface that lead to efficient bioelectrocatalysis will require additional characterization and modeling.

The bio-conjugates formed using PBSE effectively link MCO with MWCNT to facilitate DET and bioelectrocatalytic oxygen reduction. The catalytic efficiency was significantly greater than previous reports for MCO electrodes. The process provides a porous, potentially scalable, architecture that can advance bioelectrocatalytic applications. Future research will provide a deeper understanding of the attachment mechanism that directs enzyme orientation and provides guidance to optimize the interaction further. The applicability to alternative catalysts was demonstrated with two MCOs but could be extended to a wider range of biomolecules and applications.

Notes and references

† Preparation of tethered MCO–MWCNT conjugates: 10 mm^2 discs of Toray® carbon paper (TGP 090; Toray Industries, Tokyo, Japan) used as electrode base. For CNT modification of TP, 30 μL of MWCNT (1 mg mL^{-1} in *N,N*-dimethyl formamide) (Sigma, St. Louis, MO) was drop-cast onto TP and dried (50 °C, 30 min). For sidewall functionalization of CNT with PBSE, the TP/MWCNT electrodes were soaked in a PBSE solution [1-pyrenebutanoic acid succinimidyl ester; Anaspec Inc, Fremont, CA; (10 mM in *N,N*-DMF)] for 1 h at room temperature, washed thoroughly with *N,N*-DMF to remove excess PBSE and then with phosphate buffer (10 mM, pH 7). Laccase (Sigma) was dialyzed against CuSO$_4$ (10 μM) in phosphate buffer (20 mM, pH 5.8) and then stored at −20 °C until use. Protein concentrations were determined using BCA assay (BCA Protein Assay Kit, Thermo Scientific Inc. Rockford, IL). BOx (Sigma) was used as received. For protein immobilization, PBSE-modified MWCNT/TP electrodes were incubated with 0.2 mL of laccase (0.5 mg mL^{-1}) or BOx (0.2 mg mL^{-1}) for 1 h in phosphate buffer (10 mM, pH 7.0). Excess protein was removed by washing with phosphate buffer and the electrode tested immediately in an electrochemical cell. Enzyme assays: the oxidase activity of laccase was determined in phosphate buffer (100 mM, pH 6.5) using syringaldazine (21.6 μM in methanol) (Sigma) by measuring the change in absorbance at 530 nm over time. One unit of activity was defined as 1 μmol of syringaldazine oxidized by laccase in 1 minute at 37 °C. The catalytic activity of BOx was evaluated using bilirubin as substrate (0.002% in 0.2 M Tris HCl buffer, pH 8.4) by measuring the change in absorbance at 440 nm. One unit of activity was defined as 1 μmol of bilirubin oxidized by BOx in 1 minute at 37 °C. Electrochemical testing: the electrodes were held on a capped glassy carbon shaft electrode and tested in a 50 mL voltammetric cell (CH Instruments Inc, Austin, TX) with a glassy carbon counter electrode and a Ag/AgCl reference electrode (CH Instruments Inc.). Phosphate buffer (100 mM, pH 5.8, or pH 7.0 when noted) was used as the electrolyte throughout. CV scans were obtained by scanning from −0.2 V to 0.8 V at a sweep rate of 10 mV s^{-1}. Galvanostatic polarization measurements were obtained from 0 to 50 μA cm^{-2} at 10 μA cm^{-2} intervals and the voltage data points were recorded after 15 min at each step. Tafel slopes were obtained from the plots of working electrode potential versus kinetic current in the absence of convective transport.

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